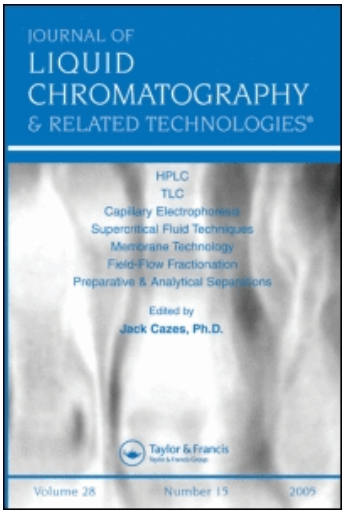

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 29 May 2003

To cite this Article Shibusawa, Yoichi , Yanagida, Akio , Shindo, Heisaburo and Ito, Yoichiro(2003) 'Separation of Apple Catechin Oligomers by CCC', Journal of Liquid Chromatography & Related Technologies, 26: 9, 1609 — 1621
To link to this Article: DOI: 10.1081/JLC-120021270
URL: http://dx.doi.org/10.1081/JLC-120021270

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, Nos. 9 & 10, pp. 1609–1621, 2003

Separation of Apple Catechin Oligomers by CCC

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ABSTRACT

Catechin oligomers with different degrees of polymerization were separated from apple condensed tannins (ACTs) by countercurrent chromatography using a type-J multilayer coil planet centrifuge. Partition coefficient values for oligomers up to pentamers of catechin and/or epicatechin were determined on hydrophilic two- or three-phase solvent systems. The best separation was achieved using a three-phase solvent system composed of hexane/methyl acetate/acetonitrile/water at a volume ratio of 1/1/1/1, which formed three layers. After the elution of monomers, dimers, trimers, and a part of tetramers using the middle

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DOI: 10.1081/JLC-120021270
1082-6076 (Print); 1520-572X (Online)

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phase, methyl acetate was used as a mobile phase to facilitate elution of the oligomers from tetramers to decamers. These oligomers eluted from the column according to their degree of polymerization. The higher polymerized oligomers over 11-mers were collected from the column with the elution by the lower phase. The masses of the fractionated oligomers were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis using 2′,4′,6′-trihydroxy acetophenone monohydrate as a matrix.

Key Words: Countercurrent chromatography; Catechin oligomers; Tannin; Three-phase-system; MALDI-TOF-MS.

INTRODUCTION

Proanthocyanidins, called condensed tannins, are widely distributed in a large variety of plants, e.g., various fruits and plant-derived foods such as wine, green tea, and chocolate. Polyphenols in unripe apples contain dihydrochalcons, phenolic acids, etc., up to 50% of the total weight, while the rest consists of catechin and/or epicatechin monomers and their oligomers (dimers to pentadecamers), which are called apple condensed tannin: ACTs. We call these oligomers in the ACTs "apple procyanidins."

Recently, procyanidins have attracted an attention in the fields of pharmacology and food chemistry because of their beneficial pharmacological effects, such as antioxidant,[1–4] antitumor,[5] antimicrobial,[6] antihypertensive,[7,8] anticancer,[9] and anti-allergy properties.[10] The prevention of arteriosclerosis,[11,12] inhibition against the activities of some physiological enzymes and receptors[13–16] and hair growth promotion effects,[17–19] were also reported. To elucidate the physiological mechanisms of action of these procyanidins and to provide these oligomers for subsequent in vivo studies, it is necessary to establish an effective separation and fractionation method.

Separations of procyanidins have been reported using normal phase,[20–22] reversed phase,[23–27] or size-exclusion[28,29] liquid chromatographic techniques. However, separation of the procyanidin oligomers beyond their pentamers has not been performed, and in addition, these chromatographic methods tend to cause irreversible adsorption of highly polymerized procyanidins (over pentamers) onto the column packing materials.

Countercurrent chromatography is a liquid–liquid partition technique that eliminates various complications arising from the use of solid supports.[30–33] In the last decade, high-speed countercurrent chromatography (HSCCC), which is the most advanced technique in terms of partition efficiency and separation time,[34] has been used for separation and purification of a wide

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variety of natural products. The recent model of HSCCC that facilitates stationary phase retention for polar solvent systems[32] is particularly useful for separation of hydrophilic apple procyanidins B and C from ACTs.[35] In the previous report,[36] higher polymerized oligomers were fractionated using a binary solvent system composed of methyl acetate and water.

In the present study, HSCCC was applied to the separation of monomers of catechin and/or epicatechin and procyanidin oligomers from ACTs, according to their degree of polymerization using three-phase solvent systems. The degree of polymerization of the procyanidin oligomers in the CCC fractions was assigned by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis.[36]

EXPERIMENTAL

Apparatus

Figure 1 shows a photograph of the type-J HSCCC centrifuge (Hitachi Tokyo Electronics Inc., Tokyo, Japan). The apparatus holds a multilayer coiled separation column and a counter-weight symmetrically at a

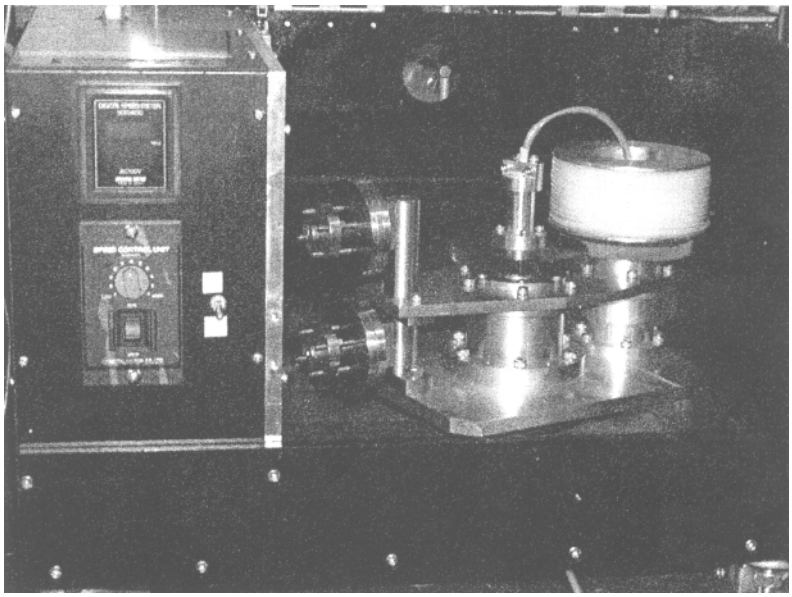

Figure 1. The photograph of the type-J multilayer coil planet centrifuge.

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distance of 10 cm from the central axis of the centrifuge. The separation column was fabricated by winding a single piece of 2.0 mm i.d. and 21 m long PTFE (polytetrafluoroethylene) tubing (Tokyo Rikakikai Co. Ltd., Tokyo, Japan) directly onto the holder hub, making four coiled layers between a pair of flanges ($\beta = 0.50$–0.62). The total capacity of the column is about 72 mL. The apparatus was regulated at 1000 rpm with a controller. In this type-J HSCCC centrifuge, the coil undergoes a planetary motion in such a way that it rotates around its axis as it simultaneously revolves around a central axis. This particular mode of planetary motion produces an efficient mixing of the two phases while retaining a sufficient amount of the stationary phase in the column.

Reagents

Hexane, methyl acetate, acetonitrile, and *t*-butyl methyl ether (*t*-BME) and methanol were all of glass-distilled chromatographic grade (Kanto Chemicals, Tokyo, Japan). Catechin and epicatechin (monomers), and their dimers, trimers, tetramers, and pentamers were purified from ACTs by normal-phase high-performance liquid chromatography.[22] Other chemicals were of reagent grade.

Preparation of Apple Condensed Tannins

The preparation of ACTs from unripe apples has been described in detail elsewhere.[28,37] About 3 kg-weight of unripe apples were homogenized in 2 L-volume of 0.1% (w/w) potassium pyrosulfite ($K_2S_2O_5$) solution, and the mixture was allowed to stand for 24 h at 4°C. The supernatant was centrifuged at 3500*g* and filtered subsequently with a glass filter funnel. The 1.8 L-volume of filtrate was applied to a Sepabeads SP-850 (Mitsubishi Kasei, Tokyo, Japan) column (28.5 × 2.5 cm i.d.). The crude apple polyphenol (CAP) fraction eluted with about 200 mL of 80% ethanol after washing the column with 300 mL distilled water. The CAP fraction was concentrated to about 65 mL in vacuo, and a 25 mL portion of the fraction was loaded on a Toyopearl HW-40EC (Tosoh, Tokyo, Japan) column (28.5 × 2.5 cm i.d.). The column was washed with 200 mL-volume of distilled water, followed by 250 mL-volume of 40% ethanol, 100 mL-volume of 60% acetone. The fractions eluted with 40% ethanol and then a 100 mL-volume of 60% acetone in water. The fractions eluted with 40% ethanol contained mainly catechin, epicatechin and their dimers, and phloretin glycosides. This fraction was passed through a Sep-Pak C18 ENV (Waters, Milford, MA) to eliminate phloretin glycoside. This eluate

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was mixed with the fractions eluted with 60% acetone, then lyophilized after evaporation of organic solvents. The collected ACTs (Fig. 3), whose structures are shown in Fig. 2, were used for CCC samples. As shown by MALDI, these ACTs were a mixture of monomeric flavan-3-ols and procyanidin oligomers ranging from dimers to pentadecamers, as shown in Fig. 3.

Measurement of Distribution Coefficient

The following two- or three-phase solvent systems were selected based on the distribution coefficient (K_D) values of catechin, epicatechin, procyanidin dimers, trimers, tetramers, and pentamers: (i) methyl acetate/water (1/1) and (ii) *n*-hexane/methyl acetate/acetonitrile/water (1/1/1/1) (volume ratio for upper, middle, and lower phase was 25/41/34). Most of the hexane is located in the upper phase. The middle phase contains a methyl acetate and acetonitrile-rich phase and the lower phase is made of water with part of acetonitrile and methyl acetate.

Each solvent mixture was thoroughly equilibrated in a test tube and each layer was separated. In the three-phase solvent systems, only the middle and lower phases were used for the determination of K_D values (the upper hexane-rich phase was not used for separation).

K_D values of samples in these solvent systems were determined as follows: About 1 mL of each phase was delivered into a test tube to which

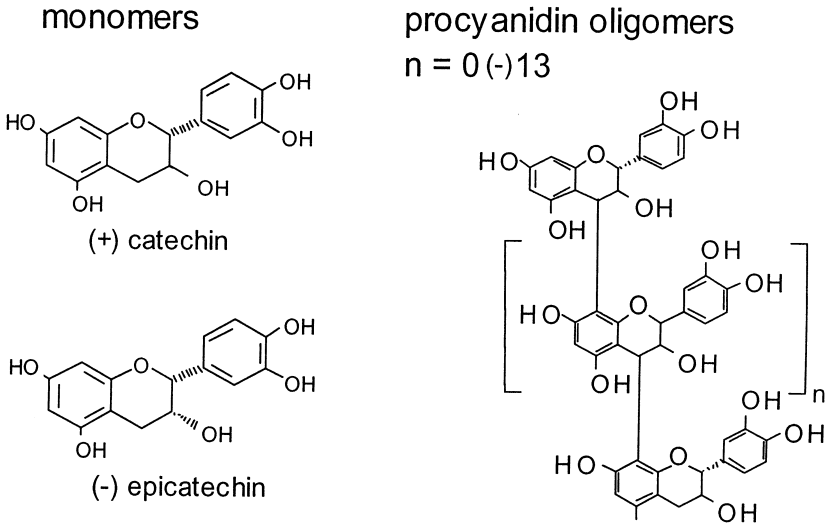

Figure 2. Structures of catechin, epicatechin, and procyanidin oligomers.

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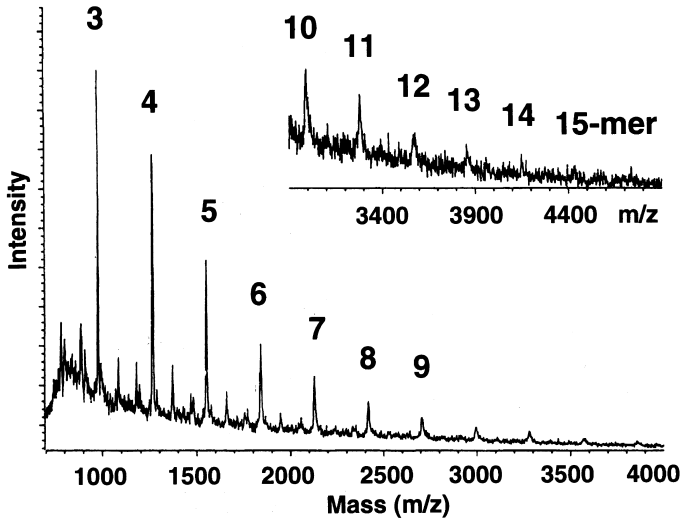

Figure 3. Matrix-assisted laser desorption/ionization time-of-flight mass spectra of apple condensed tannins used for CCC.

about 1 mg of the sample was added. The contents were thoroughly mixed and then allowed to settle at room temperature. After two clear layers were formed (the tube was centrifuged if necessary), 0.15 mL of each phase was diluted with 1.35 mL of methanol, and its absorbance was determined at 280 nm using a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). K_D was expressed as the solute concentration in the upper (middle) phase divided by that in the lower phase.

CCC Separation of Apple Condensed Tannins

The separation was performed with the three-phase solvent system using its middle and lower phases as follows: After filling the multilayer coil (72-mL capacity) with the lower aqueous stationary phase, 2 mL of the sample solution containing 102.5 mg of ACTs (5% w/w) was injected into the column using an EYELA type SV-6010 sample injector (Tokyo Rikakikai, Tokyo, Japan). Then, the middle phase was eluted through the column at a flow-rate of 1.0 mL/min at 1000 rpm revolution speed. The effluent was monitored at 280 nm, the maximum absorbance of ACTs, using a EYELA UV-9000 UV–VIS detector (Tokyo Rikakikai) and fractionated at 3 mL/tube using an LKB 2112 Redirac fraction collector (LKB Instruments, Bromma/Stockholm, Sweden). An aliquot of each fraction was diluted 10 times with methanol

and the absorbance was measured at 280 nm with a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan).

Analysis of CCC Fractions

Aliquots of CCC fractions corresponding to various portions of the major peaks were measured using MALDI-TOF-MS analysis. A 10 μL portion of CCC fraction was mixed with 5 μL of matrix solution (1% 2′,4′,6′-trihydroxy acetophenone monohydrate in acetone). The mixture (ca. 1 μL) was put on the stainless steel target tray and crystallized at room temperature. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed using a Voyager DE RP system (PerSeptive Biosystems, CA) in the linear positive-ion mode. Procyanidin oligomers eluted with the mobile phase, were ionized by a nitrogen laser (337 nm; 3 ns pulse) and accelerated at 20 kV[37].

RESULTS AND DISCUSSION

Measurement of Partition Coefficient of Apple Condensed Tannins

Because the CCC process is based on solute partitioning between mobile and stationary phases, the K_D value is one of the most important parameters to determine the peak resolution, and successful separation requires a proper choice of solvent systems. Generally speaking, a desirable range of K_D values for HSCCC is between 0.5 and 1. However, it is more important that the α value (selectivity factor, ratio of the K_D values) should be large enough to provide sufficient resolution between neighboring peaks. For separating monomers (catechin and/or epicatechin) and their oligomers according to the degree of polymerization, each component (including dimers, trimers, tetramers, and pentamers) was purified by normal phase HPLC from ACTs, and its partition coefficient value (K_D) was determined for these two polar solvent systems, as shown in Table 1. In the case of three-phase systems composed of *n*-hexane/methanol/acetonitrile/water at a volume ratio of 1/1/1/1, the middle and lower phases were used for measuring the K_D value (the upper phase, rich in hexane, was not used).

In the binary two-phase solvent system of methyl acetate/water, the K_D values of monomers (catechin and/or epicatechin) are greater than 1.0, and those of the ACTs are always smaller than 1.0, which indicates a suitable distribution of K_D values. On the other hand, the K_D values of ACTs, monomers, and oligomers (from dimers to pentamers) in the three-phase

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Table 1. Distribution coefficient of apple catechin oligomers.

		Distribution coefficient $K_D = C_U/C_L$ or C_M/C_L				
Solvent systems	Volume ratio	1 mer	2 mer	3 mer	4 mer	5 mer
Two phase systems (UP/LP)						
MeOAc : water	1 : 1	2.02	1.01	0.78	0.60	0.46
Three phase system (UP/MP/LP)						
n-Hex : MeOAc : AcCN : water	1 : 1 : 1 : 1	0.86	0.38	0.27	0.21	0.15

Note: Hex, hexane; MeOAc, methyl acetate; AcCN, acetonitrile.

solvent system composed of *n*-hexane/methyl acetate/acetonitrile/water (1/1/1/1) are all below 1 and much smaller than those obtained in the binary solvent system. However, the α values in this three-phase solvent system (middle/lower) are greater than those in the two-phase solvent system, promising an improved resolution between the neighboring peaks. The smallest K_D values of ACTs were obtained in the three-phase solvent system. This indicates that the two phases obtained from the middle and the lower phases of *n*-hexane/methyl acetate/acetonitrile/water (1/1/1/1) is relatively hydrophobic compared with the methyl acetate/water solvent system.

CCC Separation of Apple Condensed Tannins

Figure 4 shows a chromatogram obtained from ACTs using the middle phase and the lower phase of the three-phase solvent system composed of *n*-hexane/methyl acetate/acetonitrile/water (1/1/1/1). The solvent front marked SF emerged at the fifth tube. In CCC, the retention equation is $V_r = V_m + K_D V_s$, in which V_r is the compound retention volume, V_m is the mobile phase volume, V_s is the stationary phase volume (with $V_m + V_s = V_c$, the machine volume), and K_D is the distribution coefficient between the stationary phase and the mobile phase. The experiment shows a solvent front at fraction 5 that is 15 mL, so $V_m = 15$ mL, then $V_s = V_c - V_m = 72 - 15 = 57$ mL or $S_f = 79\%$. With these volume values and the K_D values of Table 1, the following Table 2 can be prepared. The inverse of the Table 1 partition coefficient should be used since the stationary phase is the polar lower phase. It gives an estimate of the retention volume and then, the number of the fraction that should contain the peak maximum. The experiment shows

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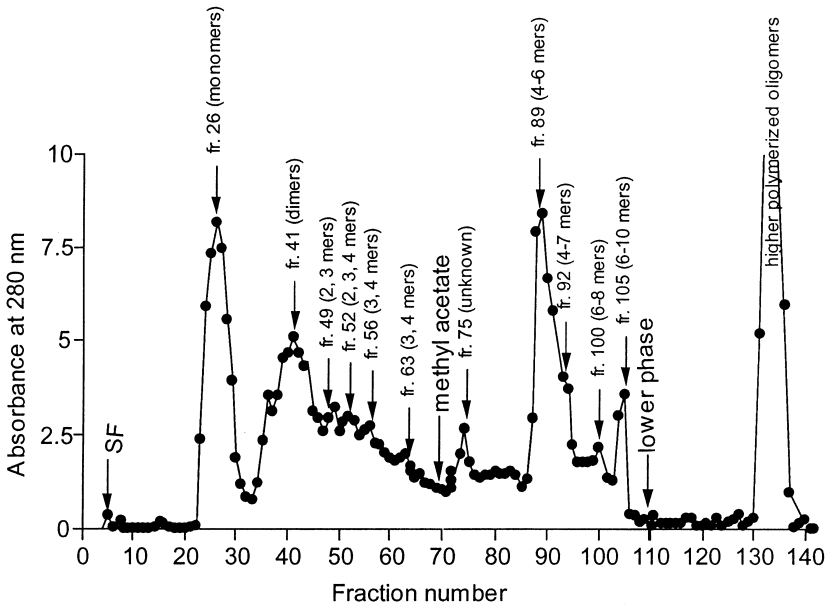

Figure 4. Countercurrent chromatographic separation of apple procyanidin oligomers according to their degree of polymerization. Experimental conditions: column is a 2.0 mm i.d. PTFE multilayer coil, 72 mL capacity; sample is the solution consisting of 102.5 mg ACTs in 1 mL each, middle and lower phase; solvent system is *n*-hexane/methanol/acetonitrile/water (1/1/1/1); stationary phase is the lower aqueous phase; mobile phase is the middle (acetonitrile methanol rich) phase; flow-rate is 1.0 mL/min; revolution speed is 1000 rpm; SF = solvent front.

that it works very well for the monomers. The dimers are collected in fractions 40–53, a little bit early. The trimers seem to be experimentally in fractions 50–66, compared to fraction 75 as calculated. Then methyl acetate, first, and the aqueous phase are used to elute the higher polymers so the calculated volumes are no longer valid.

After elution of the monomers (fr. 26), dimers (frs. 41, 49, and 52), trimers (frs. 49, 52, 56, and 63), and parts of tetramers (frs. 52, 56, and 63) with the middle phase, methyl acetate was used for the mobile phase to facilitate a rapid elution of the higher polymerized oligomers such as pentamers, hexamers, heptamers, and so on. In this way, three peaks (frs. 89, 100, and 105) and the shoulder of the main peak (fr. 92) were eluted from the column.

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Table 2. Relation between peak distribution coefficient and retention volume (peak maximum) and fraction number.

n-mer	K_D	$1/K_D$	V_r (mL)	Fraction
	MeOAc/water system			
	C_U/C_L	C_L/C_U		
1	2.02	0.50	43.2	14
2	1.01	0.99	71.4	24
3	0.78	1.28	88.1	29
4	0.60	1.67	110.0	37
5	0.46	2.17	138.9	46
	Three phase system			
	C_M/C_L	C_L/C_M		
1	0.86	1.16	81.3	27
2	0.38	2.63	165.0	55
3	0.27	3.70	226.1	75
4	0.21	4.76	286.4	95
5	0.15	6.67	395.0	132

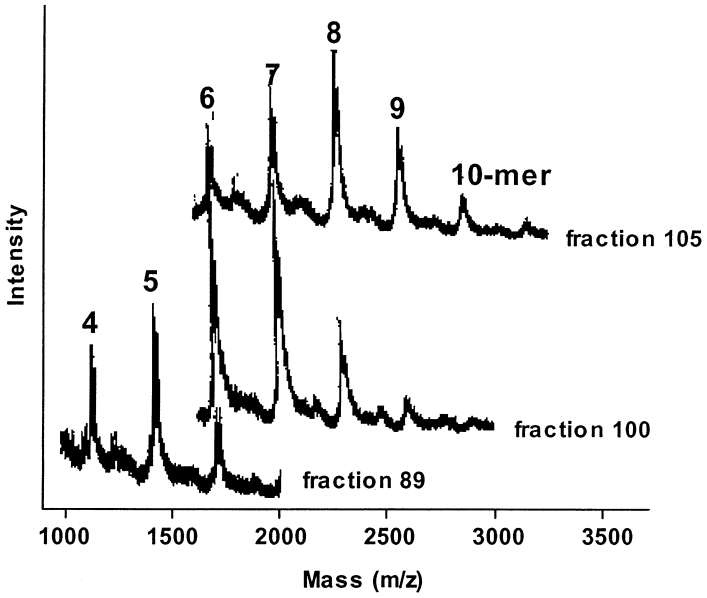

Figure 5. Matrix-assisted laser desorption/ionization time-of-flight mass spectra of CCC fractions 89, 100, and 105.

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The masses of the fractionated oligomers were determined by MALDI-TOF-MS analysis. The MALDI-TOF-MS spectra of three peak fractions, frs. 89, 100, and 105, are shown in Fig. 5. They contained the oligomer mixtures of 4–6 mers, 6–9 mers, and 6–10 mers, respectively. Fraction 92, the shoulder of the main peak, contained 4–8 mers. Interestingly, the elution order coincided with the degree of polymerization of catechin and/or epicatechin. After elution of oligomers up to decamers with the methyl acetate mobile phase, the lower hydrophilic phase was used for the mobile phase to elute the most polar component still retained in the column. The large single peak was eluted, which contained higher polymerized oligomers over 11 mers (not analyzed by MALDI-TOF-MS). The original stationary phase retained in the column prior to the application of the elution with methyl acetate was estimated as 80% of the total column capacity (72 mL). The separation was completed within 7 h.

CONCLUSION

Although HSCCC has less efficiency (plate number) than HPLC, it has more sample loading capacity and the separation can be performed without sample loss onto the solid support. The results of the present studies indicate that the method is capable of partially fractionating the apple procyanidin oligomers from ACTs according to the degree of the polymerization. The higher polymerized oligomers, which tend to be adsorbed on the silica gel column on normal phase HPLC, can be collected by the present method. We are planning to separate and purify the procyanidin oligomers from apple juice by the present method.

ACKNOWLEDGMENT

The authors are indebted to Dr. Henry M. Fales of National Institutes of Health for editing the manuscript with valuable suggestions.

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Received June 28, 2002
Accepted October 22, 2002
Manuscript 6044R

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